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Leukocytosis in Mice Following Therapy with a Novel Antitumor Agent, RA-700

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Nine daily intravenous (iv) injections of RA-700 (an antitumor cyclic hexapeptide) at doses of 2 to 6 mg/kg/day caused increases of WBC counts at 4–6 days after treatment in normal C57BL/6×DBA/2 (BDF1) mice. The percentages of neutrophils and lymphocytes were modified. There was a decrease of colony-forming units in culture (CFUc) in bone marrow to 40% of the control value on day 1 but CFUc rapidly returned to normal values on day 3. The colony-forming units in spleen (CFUs) in bone marrow decreased during treatment. On the other hand, CFUc and CFUs in spleen were increased from the initiation of treatment to the time prior to the increase of WBC count. Spleen weight increased after treatment, and histologically, increases of immature and also mature granulocytes and megakaryocytes were observed. However, RA-700 did not stimulate the progress of hematoprogenitors *in vitro*. The results indicated that RA-700 stimulates the progress of hematoprogenitors in the spleen, but this effect is probably indirect.

Key words: Leukocytosis — RA-700 — Antitumor agent — Cyclic hexapeptide

RA-700 is an antitumor cyclic hexapeptide isolated from the plant *Rubia cordifolia* by Itokawa *et al.*[1,2] RA-700 has significant, wide-spectrum antitumor activity against a series of murine tumors.[1,3] The antitumor activity of RA-700 depended on the treatment schedule, and daily treatment with RA-700 showed stronger antitumor activity than a single injection or intermittent treatment.[1,3] A previous study using intraperitoneal (ip) injection of RA-700 has indicated low hematotoxicity.[3] However, in a further study of RA-700, a transient increase of WBC counts was observed on daily intravenous (iv) injection of the compound. Here we report the effect of RA-700 on hematopoietic progenitors in normal mice.

MATERIALS AND METHODS

Mice Female specific-pathogen-free C57BL/6×DBA/2 mice (BDF1, 7 weeks old and weighing 18 to 22 g), obtained from Shizuoka Agriculture Co. Assoc., Hamamatsu, were used in the experiment.

Drug RA-700 was dissolved in a solution containing hydrogenated castor oil polyethylene glycol ester and diluted in saline. The normal BDF1 mice were given 9 daily consecutive iv injections of RA-700 at dose levels based on the optimal dose from P388 experiments. Hydrogenated castor oil polyethylene glycol ester was injected iv into control mice following the same dilution and schedule. Five mice were used for each point. Endotoxin was not detectable in RA-700 or its solvent by use of the Limulus HS-Single Test.[4]

Blood Blood was obtained with a glass capillary from the tail vein of the mice. The WBCs were stained with Türk solution, and counted with a hemocytometer. Differential leucocyte counts were performed on smear preparations stained with May-Grünwald-Giemsa.

Measurement of hematopoietic precursors Bone marrow cells were flushed out with a syringe fitted with a 26-gauge needle into RPMI 1640 medium. After counting, the cells were diluted to an appropriate concentration and used for colony-forming unit in culture (CFUc) and colony-forming unit in spleen (CFUs) assays. The spleen cells were prepared by gentle homogenization with a Potter-Elvehjem homogenizer in RPMI 1640 medium and assayed for CFUc and CFUs.

CFUc was assayed by the technique of Bradley and Metcalf.[5] One milliliter of 0.3% agar in RPMI 1640 medium supplemented with 20% fetal calf serum, containing 5×10^4 cells for bone marrow cells or 2.5×10^5 cells for spleen cells, was plated in 35 mm Petri dishes. A 10% peritoneum-conditioned medium was used as a source of colony-stimulating activity.[5] Triplicate plates for each dose were incubated at 37°C in 5% CO_2 in humidified air, and the colonies were counted 7 days later. The CFUs was determined according to the method of Till and McCulloch.[6] Five hundred thousand spleen cells or 10^5 bone marrow cells were injected iv into lethally irradiated recipient mice, and the spleen colonies were counted 10 days later. Recipient mice for CFUs assay were irradiated (750 R) before cell injection.

Histology The spleen was fixed in 10% formalin and histological sections were routinely stained with hematoxylin and eosin.

RESULTS

Effects on blood Nine daily iv injections of RA-700 at a dose of 2 to 4 mg/kg/day produced significant increases in the lifespan of P388-bearing mice (data not shown). Therefore, normal BDF1 mice were injected iv with doses of 2 to 6 mg/kg/day of RA-700. The effects of RA-700 on WBC count are shown in Fig. 1. WBC counts were reduced during treatment at all doses of RA-700, and then increased transiently to about 15,000 to 30,000/mm^3 at day 14, 5 days after the last injection and recovered to the normal range thereafter. The percentages of neutrophils and lymphocytes were modified (Table I). On day 12 and day 14, the WBC counts showed both neutrophil and lymphocyte increases. A marked increase of neutrophils was observed at 6 mg/kg/day. The compound did not affect RBC or platelet counts (data not shown).

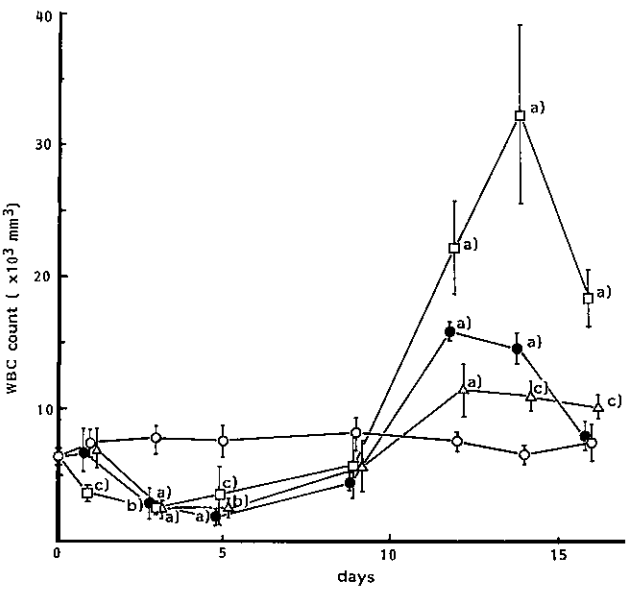

Fig. 1. Effect of 9 daily iv RA-700 injections on the number of WBC. ○, control; □, RA-700 6 mg/kg/day; ●, RA-700 4 mg/kg/day; △, RA-700 2 mg/kg/day. Bars, SD. a) $P<0.005$. b) $P<0.01$. c) $P<0.1$.

Effects on bone marrow As shown in Fig. 2-A, a decrease in the femur cellularity was caused by iv injection of RA-700, and it persisted during treatment but recovered thereafter. The effect of RA-700 on bone marrow CFUc is also shown in Fig. 2-B. After 1 injection of RA-700 at the dose of 4 mg/kg, the CFUc of treated mice was about 40% of that of control mice, and returned to the control level on day 3. On the other hand, a decrease of CFUs in the bone marrow persisted during treatment because there was less femur cellularity, although an increase of CFUs was observed during treatment (Fig. 2-C).

Effects on spleen Spleen weight increased after treatment, resulting in increased cellularity of the spleen (Fig. 3-A). Histologically, expansion of the red pulp and increase of immature and mature granulocytes and megakaryocytes were observed (data not shown). The effects of RA-700 on the spleen CFUc and CFUs are also shown in Fig. 3-B and C. A marked increase of CFUc and CFUs in the spleen was observed, with a maximum prior to the maximum of WBC counts. The CFUc in the spleen was slightly decreased on day 1, and increased to about 50 times that of the control on day 7 to day 10. The CFUs in the spleen also increased gradually during treatment, and colonies were observed in the overall spleen on day 10. When spleen cells were exposed to RA-700 at non-cytotoxic doses (0.001 and 0.01 μg/ml) for 7 days *in vitro*, the colony number did not increase in comparison with the control (data not shown).

Table I. Effect of RA-700 on Neutrophil and Lymphocyte Counts

Dose (mg/kg/day)			Day 9	Day 12	Day 14	Day 16
RA-700	6	N	1588±658	8360±1341[a]	24120±4489[a]	10878±1314[a]
		L	3969±1645	13420±2153[b]	10230±2144[c]	5983±723
	4	N	865±150	4611±218[a]	5184±382[a]	3252±380[a]
		L	3640±632	10653±503[a]	9072±668[a]	4146±485
	2	N	1783±605	3286±592[c]	3465±406[c]	2909±252[a]
		L	3910±1326	8044±1448	7364±867[c]	6118±531
Control		N	2000±248	1360±102	780±907	1026±203
		L	6000±743	6160±462	5395±410	6157±1218

RA-700 was given iv to BDF1 mice once daily for 9 consecutive days. Differential leucocyte counts were performed on smear preparations. N: Neutrophil/mm^3. L: Lymphocyte/mm^3.
a) $P<0.005$. *b*) $P<0.01$. *c*) $P<0.1$.

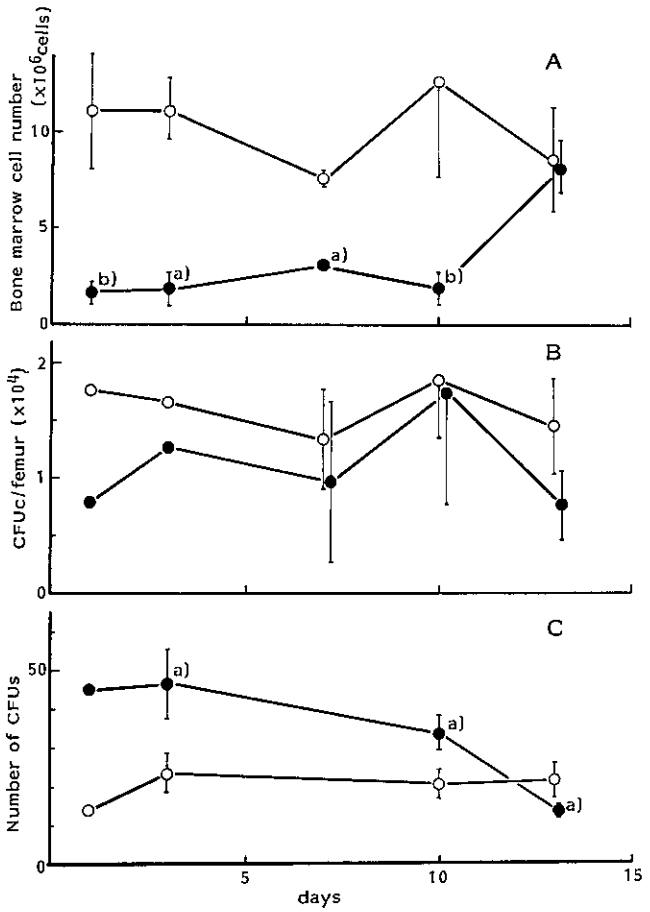

Fig. 2. Effect of 9 daily iv RA-700 injections (4 mg/kg/day) on cellularity (A), CFUc (B) and CFUs (C) in bone marrow of normal BDF1 mice. ○, control; ●, RA-700. Bars, SD. a) $P<0.005$. b) $P<0.1$.

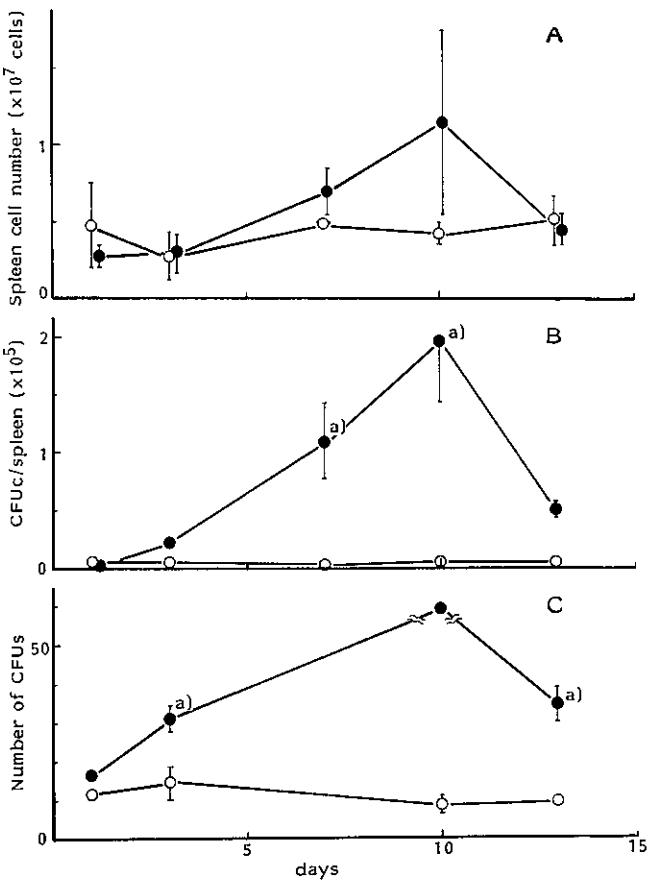

Fig. 3. Effect of 9 daily iv RA-700 injections (4 mg/kg/day) on cellularity (A), CFUc (B) and CFUs (C) in spleen of normal BDF1 mice. ○, control; ●, RA-700. Bars, SD. a) $P<0.005$. b) $P<0.1$.

DISCUSSION

As reported in this study, nine daily iv injections of more than 2 mg/kg/day of RA-700 caused a decrease in the WBC counts during treatment and an increase after treatment. This change suggests that RA-700 accelerated release of cells from the marrow, since it decreased marrow cellularity at 2 or 6 h after injection (data not shown). Furthermore, the compound also decreased CFUc and CFUs in bone marrow. On the other hand, CFUc and CFUs in the spleen were gradually increased and the maximum value was observed a few days before the increase of WBC counts. An increased spleen weight was also observed after treatment. The increase in the number of immature and mature granulocytes was histologically confirmed. The results indicate that the major hematopoietic site changed from bone marrow to spleen, and the proliferation of hematoprogenitors in spleen was enhanced by the treatment with RA-700.

It has been reported that irradiation and cyclophosphamide cause rises in serum colony-stimulating factor (CSF) leading to the recovery of granulopoietic tissue from hemopoietic damage.[7] Since a reduction of WBC counts was observed during treatment, the leukocytosis with RA-700 might be a rebound from bone marrow suppression. However, as shown in Fig. 3, CFU in the spleen increased from the initiation of RA-700 treatment. The effects of RA-700 are very similar to these seen following the injection of endotoxin. Since endotoxin was not present in the compound or its solvent, leukocytosis following RA-700 therapy seems to be caused by the compound itself. The marked increase of WBC count with RA-700 may have been caused by a CSF-like function of the compound or enhanced CSF

production rather than only rebound of bone marrow suppression. But the colony number with RA-700 showed no significant difference from the control when spleen cells were exposed directly to RA-700 at a non-cytotoxic dose *in vitro*. Therefore, RA-700 seems to act indirectly on hematoprogenitors. Since endotoxin causes the release of mediators such as CSF and interleukin-1 from macrophages and other cells,[7] RA-700 might also cause the leukocytosis by a similar mechanism. Further study of the mechanism of RA-700 action on hematoprogenitors is required.

In this study, we determined only granulocyte-macrophage colony-forming units (GM-CFU). Although CFUs in bone marrow decreased during treatment, bone marrow CFUc decreased only at day 1 and recovered thereafter. Both CFUs and CFUc in the spleen increased during treatment with RA-700. An increase in lymphocytes was also observed. The histological study on the spleen showed that not only granulocytes, but also megakaryocytes were increased. Therefore, further studies are needed to determine whether stem cells or committed progenitors are stimulated by RA-700 treatment.

RA-700 has a wide spectrum against a series of murine tumors.[1] From the viewpoint of cancer treatment, it may be useful that RA-700 has not only antitumor activity but also the above-mentioned activity. Although it is unclear whether the increase of WBC by RA-700 treatment has a physiological role, we observed that the combination of RA-700 with anticancer agents which have hematotoxicity, such as cyclophosphamide and 3-((4-amino-2-methyl-5-pyrimidinyl)ethyl)-(2-chloroethyl)-1-nitrosourea (ACNU), produced a significant enhancement of antitumor activity.[8] Since the combination therapy did not enhance the activity of any of the drugs *in vitro*, it appears that RA-700 exhibits indirect activity in tumor-bearing mice. Although further study of RA-700 action on hematoprogenitors in tumor-bearing mice is required, RA-700 may be clinically useful in combination with anticancer agents which have hematotoxicity.

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